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Bracken fern-induced malignant tumors in rats: absence of mutations in *p53*, *H-ras* and *K-ras* and no microsatellite instability

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Abstract

Bracken fern (genus *Pteridium*) has been shown to induce tumors in domestic and experimental animals. Epidemiological studies have also shown an association between human exposure to bracken toxins and increased risk for the development of upper gastrointestinal tract tumors. Our aim in this study was to investigate possible genomic alterations in bracken fern-induced tumors of experimental animals searching for molecular markers that might be used for human epidemiological studies. Using human colorectal carcinogenesis as a molecular model, we examined eight malignant bracken fern-induced tumors of rats for mutations in the genes associated with the "classic pathway" of colorectal cancer, i.e. *p53* and *ras*, and also in the "mutator pathway" by evaluating microsatellite instability. Exons 5–9 of the *p53* gene and exons 1 and 2 of the *K-ras* and *H-ras* genes were examined by DNA sequencing and no mutations were found in any of the eight tumors. Amplification of five previously validated microsatellite loci (one with mono-, three with di- and one with tetra-nucleotide repeat motifs) in the malignant tumors and in the surrounding normal tissue did not reveal any instability. The involvement of epigenetic alterations or of mutations in other tumor suppressor genes or oncogenes should be further investigated in the search for human epidemiological markers.

Keywords: Bracken fern carcinogenesis; Microsatellite instability; *ras*; *p53*; Mutation

1. Introduction

Bracken fern (genus *Pteridium*) has been described as one of the most common plants on the planet [1], and it apparently is the only one that causes tumors naturally in animals. The toxicity and carcinogenicity of bracken fern to domestic and experimental animals has been extensively described [2–4]. In mice, the bracken fern can induce leukemia or solid tumors of the stomach, while in rats it is associated with cancer in the ileum, urinary bladder or mammary gland [3]. Bracken fern is grown commercially for human consumption in several regions, including Japan, Canada, Siberia, northeastern US and it is also eaten by humans in the Ouro Preto region of the state of Minas Gerais in Brazil [3,5]. Moreover, ingestion of bracken toxins can also occur indirectly through dairy products from

Abbreviations: DB: dry bracken; HNPCC: hereditary non-polyposis colorectal cancer; MSI: microsatellite instability; MSI-H: high frequency microsatellite instability; WCB: water from cooked bracken

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contaminated cattle milk or by aspiration of bracken spores in the spring [5–11]. Epidemiological studies have shown a suggestive association between human exposure to bracken and increased risk of tumors in the upper gastrointestinal tract [5,6,8,12]. There is an urgent need for molecular markers that may confirm or not the carcinogenicity of bracken fern in humans.

Cancer is thought to emerge as a result of a "genetic instability" causing the continuous occurrence of genetic and epigenetic changes that enable the cell to escape from normal cellular and environmental controls [13]. In human colorectal cancer, two different pathways to genomic instability mechanisms have been proposed [14]. One, the so-called "classic pathway", is the most common and depends on multiple additive mutational events (germline and/or somatic) in tumor suppressor genes and oncogenes [14]. A series of studies have established that in this pathway, additive mutations in the *K-ras* oncogene, together with the inactivation of the tumor suppressor genes *p53*, *DCC*, and *APC*, occur in most patients and are implicated in the stepwise transformation of the normal mucosa into a malignant tumor [15]. On the other hand, the "mutator pathway", characterized by the presence of microsatellite instability (MSI), depends on early functional loss of the mismatch repair system leading to accumulation of gene mutations in critical target genes and progression to malignancy. Microsatellite instability is a hallmark of colonic and endometrial tumors in patients with hereditary non-polyposis colorectal cancer (HNPCC), but it also occurs in a substantial proportion (15–25%) of sporadic colorectal cancers [14].

In this work, we have systematically evaluated the possibility that the "classic" or the "mutator" pathways were involved in intestinal tumorigenesis induced by bracken fern in rats. We searched for mutations in the *K-ras* and *H-ras* oncogenes and in the *p53* tumor suppressor gene by nucleotide sequencing and evaluated the degree of genetic stability of five different microsatellite loci.

2. Material and methods

2.1. Tumor induction with bracken fern

Bracken fern (*P. aquilinum* var. *caudatum* subs. *arachnoideum*) young fronds freshly collected in Ouro Preto neighborhoods (Minas Gerais, Brazil), were cut into small pieces (2–3 cm), dried in an air–draft oven at 60 °C for 24 h and ground to a fine powder that was stored at −20 °C. Alternatively, the young fronds were cut into small pieces, boiled in water three times (10–12 min each) and the water from the three soakings was collected, pooled and stored at −20 °C until use. Wistar female rats, 45 days old, were separated in three groups. One group was fed with a standard diet containing 10% (w/w) of the dried bracken powder *ad libitum* ($n = 6$). Another group received standard diet and the water obtained from boiling bracken as the only source of water *ad libitum* ($n = 20$). The last group (control) received normal diet and water for the whole experimentation time ($n = 13$). The experimental animals were sacrificed (from 15 to 24 months) when they presented signals of poor health conditions (hemorrhages, colorless eyes, loss of sphincter control). The control group animals were sacrificed after the last experimental animal. After sacrifice, the animals were carefully examined for the presence of tumors, especially, in the gastrointestinal tract and urinary bladder. Fragments of normal tissue and lesions of these organs were collected, fixed in absolute ethanol and kept at −20 °C until DNA extraction. Matched tissue fragments were fixed in 10% formalin, sectioned and stained with hematoxylin and eosin.

2.2. DNA extraction

Fragments (10–30 mg) of the tumors and of adjacent normal tissue were removed from the ethanol, microdissected to contain at least 80% of neoplasic tissue and DNA was extracted using a standard phenol/chloroform procedure after digestion with proteinase K [16].

2.3. PCR amplification of the p53, K-ras and H-ras genes

A mix of *Taq* DNA polymerase and of the proofreading *Pfu* DNA polymerase (15:1) was used in all PCR reactions. Exons 5,6,7–8 (together) and 9 of the *p53* gene and exons 1 and 2 of *K-ras* and *H-ras* genes were amplified from 20 to 50 ng of DNA samples. The sequence of the primers used, their annealing temperatures and their amplicon sizes are listed in Table 1. The reaction tube contained

Table 1
Sets of primers used for amplification of the regions sequenced, respective annealing temperatures and size of the products obtained

Gene	Region	Primers	Size (bp)	Annual temperature (°C)
p53	Exon 5	1st PCR		
		Forward: gacctttgattctttctcctctcc	265	60
		Reverse: gggagaccctggacaaccag		
		2nd PCR		
		Forward M13: gtaaaacgacggccagtattctttctcctctcctacag	265	63
		Reverse: gggagaccctggacaaccag		
		Reverse M13: caggaaacagctatgacagttctaaccccacagcagtg	265	63
		Forward: gacctttgattctttctcctctcc		
	Exon 6	1st PCR		
		Forward: ctggttgtccagggttctcc	294	60
		Reverse: cccaacctggcacacagctt		
		2nd PCR		
		Forward M13: gtaaaacgacggccagtcccggcctctgacttattctt	294	65
		Reverse: cccaacctggcacacagctt		
		Reverse M13: caggaaacagctatgaccctggcacacagcttcactac	294	65
		Forward: ctggttgtccagggttctcc		
	Exons 7 and 8	1st PCR		
		Forward: cttactgccttgtgctgtgc	351	56
		Reverse: taatccaataataaccttgg		
		2nd PCR		
		Forward M13: gtaaaacgacggccagtgtgctgtgcctcctcttgt	351	60
		Reverse: taatccaataataaccttgg		
		Reverse M13: caggaaacagctatgacatccaataataaccttggtac	351	60
		Forward: cttactgccttgtgctgtgc		
	Exon 9	Forward: tctgtcctacttcatccttg	144	60
		Reverse: aggtcactcacctggagtga		
K-ras	Exon 1	1st PCR		
		Forward: gcctgctgaaaatgactgag	116	57
		Reverse: cgtaggatcatattcatcca		
		2nd PCR		
		Forward M13: gtaaaacgacggccagtgcctgctgaaaatgactgag	133	60
		Reverse: cgtaggatcatattcatcca		
		Reverse M13: caggaaacagctatgacagtgattctgaattagctgt	110	60
		Forward: gcctgctgaaaatgactgag		
	Exon 2	1st PCR		
		Forward: gactcctacaggaaaca	138	60
		Reverse: ggcaaatacacaaagaaagc		
		2nd PCR		
		Forward M13:gtaaaacgacggccagtgactcctacaggaaacaagtag	155	57
		Reverse: ggcaaatacacaaagaaagc		
		Reverse M13: caggaaacagctatgacgtaattcatggagaaacctg	134	57
		Forward: gactcctacaggaaaca		
H-ras	Exon 1	Forward: gtttggcaacccctgtagaa	132	57
		Reverse: ctatagtgggatcatactcg		
	Exon 2	Forward: aggtagtcattgatggggag	140	57
		Reverse: ggacttggtgttgttgatgg		

20–25 pmol of each primer, 2.5 mM dNTP, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100 and 10 mM Tris–HCl, pH 8.4 in a 10 μl final volume. After an initial denaturation for 5 min at 95 °C, the reaction was allowed to occur for 35 cycles of 1 min at each respective annealing temperature, 1 min at 72 °C and 1 min at 95 °C followed by a 5 min final extension.

2.4. Cloning and sequencing of PCR products

The products obtained from *p53* exon 9 amplification and from *H-ras* exons 1 and 2 were purified using the Wizard PCR Prep purification kit (Promega) and inserted into a pUC vector using the Sure Clone® kit (Amersham, Pharmacia Biotech) or into a pGEM-T vector using pGEM®-T Easy Vector system (Promega). These vectors were used to transform competent DH5α cells. Five transformed clones were randomly selected, grown and the plasmids were purified with the SV Plus Miniprep kit (Promega). Both strands of each plasmid were sequenced with M13 fluorescent forward or reverse primers (1 pmol), using the Thermo Sequenase fluorescent labeled primer cycle kit (Pharmacia Biotech) and an automatic DNA sequencer (Alfwin, Pharmacia Biotech). The sequences obtained were edited and aligned using the software Alfwin (Pharmacia Biotech).

2.5. PCR product direct sequencing

The products of the first PCR from exons 5–8 of *p53* and exons 1 and 2 of *K-ras* were reamplified in an hemi-nested format using an internal primer designed with a 5′ tail composed either of the M13 universal or the M13 reverse primer (Table 1). Each fragment of the first PCR was reamplified twice, so that each time the forward or the reverse nested primer containing the "M13 tail" was used. The reaction protocol was basically the same as for the first PCR, but in a 50 μl volume. The products of this second amplification were purified using the Wizard PCR Prep purification kit (Promega). Each PCR product obtained in the second amplification was then submitted to a sequencing reaction using the M13 fluorescent forward or the reverse primer (1 pmol), depending on which strand was been sequenced, using the Thermo Sequenase fluorescent labeled primer cycle kit (Pharmacia Biotech) and an automatic DNA sequencer (ALF, Pharmacia Biotech). The sequences obtained were edited and aligned using the software Alfwin (Pharmacia Biotech). Each experiment was done in duplicate.

2.6. Microsatellite instability

Five microsatellite loci (*IGHE*, *PRLR*, *ADRB2*, *PBPC2* and *IVD*) were simultaneously amplified by PCR from 20 ng of DNA from 7 malignant tumors and normal tissue adjacent to the tumors. The first four of these loci had already been previously used for detection of microsatellite instability in rat tumors [17]. The primer sequences and PCR conditions were as described by Serikawa et al. [18]. The PCR products were analyzed in an 8% polyacrylamide gel electrophoresis containing 7 M urea and stained with silver salts [19].

3. Results

3.1. Induction of tumors

Tables 2 and 3 present the results for the incidence and distribution of the proliferative lesions found in rats after prolonged treatment with the water from cooked bracken (WCB) or with dry bracken (DB)

Table 2
Incidence and histological types of proliferative lesions found in rats treated with WCB, DB in the diet or without treatment (control)[a]

Site	Type of lesion	Control ($n = 13$)	WCB ($n = 20$)	DB ($n = 6$)
Ileum	Hyperplasia	0	4	2
	Adenoma	0	13	3
	Adenocarcinoma	0	5	3
	Carcinoma	0	1	0
Bladder	Hyperplasia	0	4	1
	Papilloma	0	5	2
	Adenoma	0	1	0
	Carcinoma	0	1	0
Other	Miscellaneous	1[b]	8[c]	0

[a] Number of rats with each type of lesion is indicated.
[b] One animal presenting one isolated cystadenofriboma.
[c] Two animals presenting cervical polyp, four animals presenting isolated cystadenofriboma, one animal presenting cervical fibroma and one animal presenting uterus leiomyoma.

Table 3
Distribution of lesions among rats treated with WCB, DB in the diet or without treatment (control)[a]

Group (*n*)	Ileum	Bladder	Other	Multiple sites	No. lesion
Control (13)	0	0	1	0	12
WCB (20)	17	8	8	10	0
DB (6)	4	2	0	2	2

[a] Number of rats presenting lesions in each site is indicated.

in the diet. Several lesions, varying from mucosal hyperplasia to adenocarcinomas and carcinomas, were found in both groups of bracken treated animals. One animal from the control group presented one isolated peritoneal benign cystadenofibroma, a lesion that was also found in 4 rats treated with WCB. The other 11 rats from the control group did not present any lesions. Ileal adenocarcinomas or carcinomas were found in six out of twenty rats (30%) treated with WCB and in three out of six rats (50%) treated with DB. Only one of the treated rats presented a malignant tumor in the urinary bladder (transitional cell carcinoma). Seven ileal adenocarcinomas and the single bladder carcinoma were used for the genomic studies.

3.2. Nucleotide sequencing of the H-ras, K-ras and p53 genes

Five clones of the PCR amplification product of exons 1 and 2 of the *H-ras* gene from each tumor and from the matching adjacent normal tissue were sequenced in both directions. No mutations were found in exon 1. However, four out of eight tumor samples analyzed showed a heterozygous mutation in codon 53 (CTG → ATG), while three of these samples also showed a nucleotide substitution in the codon 76 (GAG → AAG). However, the normal tissue sample also showed both heterozygous mutations.

The regions encompassing codons 4–20 (exon 1) and codons 52–76 (exon 2) of *K-ras* gene were directly sequenced for the same tumor samples above. No mutations were found in tumors or in the normal tissues. Likewise, no mutations were seen when both DNA strands from entire exons 5–8, and intron 7 of *p53* gene were directly sequenced from PCR products obtained from the same tumor samples used above. Exon 9 was sequenced after cloning and again, no mutation was found.

3.3. Microsatellite instability

Five loci of rat microsatellites were amplified from DNA obtained from seven pairs of normal and malignant tumor tissue samples (six ileum adenocarcinomas and one urinary bladder carcinoma). The loci were located in five different chromosomes [17,18]. None of the samples examined presented microsatellite instability at any of the loci analyzed in denaturing polyacrylamide gel electrophoresis.

4. Discussion

Previous studies with dry bracken fern (*P. aquilinum* subsp. *caudatum* var. *arachnoideum*) from the Ouro Preto region of the state of Minas Gerais in Brazil had shown the induction of ileum and bladder malignant tumors in rats after prolonged treatment [20–23]. These findings were largely confirmed in the present work, where we found premalignant and malignant lesions of the histologic types of bracken induced tumors in ileum and urinary bladder, in rats treated either with DB in the diet or with the WCB. In our study, we observed a preponderance of ileal rather than bladder neoplasias, and we observed more benign (adenoma or papilloma) rather than malignant tumors compared with previous studies using the same bracken variety [21,22]. We believe that these differences may be due to seasonal variation of the toxic components of the bracken ferns, as has been demonstrated elsewhere [24].

In humans, it has been suggested that there are two major pathogenetic mechanisms leading to colorectal cancer development [14]. The first mechanism, the so-called “classical pathway”, involves cumulative mutations in tumor suppressor genes and oncogenes. The second mechanism, so-called “mutator pathway” involves mutations or altered expression of the DNA mismatch repair genes and is primarily manifested by microsatellite instability. In order to ascertain whether bracken fern-induced malignant tumors in rats were following either of these pathways, we sequenced the tumor suppressor gene *p53* and the oncogenes *H-ras* and *K-ras* in eight malignant bracken fern-induced

tumors, and also screened the lesions for microsatellite instability.

Most missense *p53* mutations in cancer are clustered in exons 5 through 8, which include highly conserved sequence blocks [25] and a sequence-specific DNA binding domain [26]. Inactivating mutations in these domains result in a loss of the growth suppression function of the p53 protein. In the present work, no mutation was found in these regions in the malignant ileum and bladder tumors analyzed. In concordance with our results, Shahin et al. [27] did not find any mutations in exons 5–7 of the *p53* gene in mammary gland tumors induced in rats by activated ptaquiloside, one of the proposed main carcinogens in bracken fern.

Another important gene mutated in the "classic pathway" for the colorectal cancer development is *K-ras*. Thus, we decided to sequence, in the same samples we had sequenced the *p53*, the codons in the exons 1 and 2 of *K-ras* where activating mutations occur. Since mutations in codons 61 and 59 of the *H-ras* gene had been described in ileum tissue from calves feeding on bracken [28], we decided to also sequence regions of the exons 1 (codons 1–30) and 2 (codons 51–70) of this gene in the same samples as above. In the present work, we did not find any mutations in the exons 1 and 2 of the *K-ras* and *H-ras* genes derived from eight ileum and bladder malignant lesions. We detected two apparent genetic polymorphisms in the exon 2 of *H-ras* gene of ileum tissues from bracken treated rats. This polymorphism consisted in a C → A transition that means a change of a Leu residue for a Met residue in codon 53 and a G → A transition in codon 76 implying in a change of Glu for Lis. These positions are not related with the positions where activating mutations usually occur in *ras* genes.

In ileum tissue from calves feeding on bracken, codon 61 showed an A → C transversion the second nucleotide and codon 59 presented different mutations in the third base of the *H-ras* gene [28]. However, in the present study we did not find mutation in these codons of the *H-ras* gene. In another study, the same researchers found a third base mutation in codon 58 and a first base mutation in codon 59 in normal mammary gland tissue but not in mammary tumors from rats treated intravenously with activated ptaquiloside [27]. None of these results was confirmed by the present work. We believe that the mutations found in the *H-ras* gene of the rat mammary gland are not causally related to the tumors, since they were detected in normal tissues, but not in the tumors themselves.

Since we did not find mutation in the *p53* and *ras* genes in the malignant tumors that we obtained after bracken fern treatment of the rats, we decided to study the so-called "mutator pathway". To test this hypothesis, we have searched seven of the malignant tumors (from which we had matching normal tissue) for MSI. The MSI is caused by a failure of the DNA mismatch repair system to repair errors that occur during the replication of DNA and is characterized by the accelerated accumulation of single nucleotide mutations and alterations in the length of simple, repetitive microsatellite sequences that occur ubiquitously throughout the genome. We analyzed five rat microsatellite loci: *IGHE*, *ADRB2*, *PRLR* and *PBPC2* (di- or tetra-nucleotide loci) that had been previously analyzed in colon tumors induced by heterocyclic amines in rats and shown to be suitable for screening for instability [17]. The *IVD* locus was chosen because it is a poly A repeat with 20 nucleotides [18], similar to the *BAT26* locus that has been proposed as the most sensitive marker for MSI in human tumors [29]. Our results show convincingly that, bracken fern induced malignant tumors in rats do not present genuine microsatellite instability. For humans, a workshop of the National Cancer Institute established international criteria for determination of microsatellite instability in colorectal cancers and validated a panel of five microsatellites [30]. The same observations are true for gastric tumors, where it has been proposed that tumors with high frequency MSI (MSI-H) can be detected analyzing only the *BAT26* locus [31]. Thus, we believe that the markers we used were enough to reveal if any tumor presented MSI-H.

To ascertain, whether bracken fern indeed causes malignant digestive tract tumors in humans, we need molecular markers, preferably characteristic of bracken fern carcinogenicity. Unfortunately, in spite of using the most sensitive techniques available, we did not detect any such genomic lesions in bracken fern-induced malignant tumors of rats. Perhaps, continuing work concentrating on epigenetic or chromosomal alterations, rather than mutation detection, may provide these much needed molecular markers.

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